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Evidence for the Function of P^1, P^4-Diguanosine 5′-Tetraphosphate in the Development of *Artemia salina**

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J. S. CLEGG,‡ A. H. WARNER,§ AND F. J. FINAMORE

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

SUMMARY

Acid extracts of cysts and newly hatched nauplii of *Artemia salina* contain large amounts of guanine derivatives but only very small amounts of adenine compounds. As the nauplii undergo further differentiation, the total purine concentration remains constant, but the total adenine concentration increases. The increase in total adenine equals the decrease in total guanine. Since the nauplii are incapable of synthesizing purines *de novo*, a direct conversion of guanine compounds to adenine compounds must occur.

Furthermore, P^1, P^4-diguanosine 5′-tetraphosphate appears to serve as a direct precursor of adenosine triphosphate, its guanine moiety being converted to adenine and its pyrophosphate bonds being conserved in the transition. The other guanine-containing compounds of the acid-soluble pool appear to serve as a source of nucleic acid guanine.

Finamore and Warner (1) have found large quantities of a unique pyrophosphate anhydride—P^1, P^4-diguanosine 5′-tetraphosphate—in encysted embryos of the brine shrimp *Artemia salina*. They have suggested that the pyrophosphate linkage formed between 2 molecules of guanosine 5′-diphosphate conserves vital high energy phosphate bonds during the prolonged quiescent period and that the compound may be a primary source of energy for further development of the organism.

In addition, Warner and Finamore (2) have isolated and partially purified from the same organism P^1, P^4-diguanosine 5′-tetraphosphate asymmetrical pyrophosphohydrolase, an enzyme that specifically hydrolyzes diguanosine tetraphosphate[1] to equimolar quantities of GMP and GTP.

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‡ This work was done on Oak Ridge Associated Universities Research Participantship at Oak Ridge National Laboratory. Present address, Laboratory for Quantitative Biology, University of Miami, Coral Gables, Florida.

§ Present address, Department of Biology, University of Windsor, Windsor, Ontario, Canada.

[1] The abbreviations used are: diguanosine triphosphate, P^1, P^3-diguanosine 5′-triphosphate; diguanosine tetraphosphate, P^1, P^4-diguanosine 5′-tetraphosphate.

Finally, Warner and Finamore (3) have described changes in the acid-soluble guanine-containing nucleotides during the early development of *Artemia salina* and have shown that the concentration of diguanosine 5′-tetraphosphate remains constant throughout embryonic development and hatching of the nauplius but that afterward it starts to decline precipitously. An important question raised by this observation, and one with an answer which may well elucidate the function of this compound, is: What is the fate of the guanine derivatives from diguanosine tetraphosphate after hatching of the nauplius?

We suggest that the guanine derivatives from diguanosine tetraphosphate may undergo one or more of the following transformations.

1. The guanine residues may be excreted as such or may be completely degraded and then excreted by the nauplii.
2. They could appear as GMP and GTP (or their hydrolytic products) by virtue of the action of the asymmetrical pyrophosphohydrolase on diguanosine tetraphosphate.
3. The guanine components could appear as nucleic acid guanine.
4. Finally, the components of diguanosine tetraphosphate (or their hydrolytic products) may be converted to different but related compounds that are retained by the nauplii.

This paper presents evidence that diguanosine tetraphosphate serves as a direct precursor of ATP, its guanine moiety being converted to adenine and its pyrophosphate bonds being conserved in the transition.

EXPERIMENTAL PROCEDURE

Incubation of Cysts and Collection of Nauplii for Acid-soluble Nucleotide Studies—Dry cysts from the Great Salt Lake (Sanders Company, Ogden, Utah) were sterilized by immersion (with stirring) in 7% antiformin solution for 30 min in the cold. They were then rinsed thoroughly with cold distilled water, filtered through a coarse fritted glass funnel to remove the water, and weighed. Ten-gram portions were then placed in Fernbach flasks containing 500 ml of sterile artificial seawater and were gently shaken on a platform rotator at 30° for the desired length of time. The developing embryos were collected by filtration through a coarse fritted glass funnel and were used directly for preparation of the acid-soluble fraction. For collection of

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hatched nauplii, the entire contents of the flasks were poured into large separatory funnels, and after a few minutes the nauplii that gathered at the stopcock were collected. This procedure was repeated until the nauplii were free of empty egg shells and debris. The nauplii were then pipetted into crystallizing dishes which contained 100 ml of artificial seawater fortified with 5 g of NaCl, 100,000 units of penicillin, and 10 mg of streptomycin. At the desired number of days after hatching, the nauplii were collected on cloth filter supports, washed thoroughly with sterile artificial seawater, and used directly for preparation of the acid-soluble fraction.

Preparation of Acid-soluble Fraction—Encysted embryos were ground in 20 ml of 1 N $HClO_4$ in a motor-driven mortar and pestle for 20 min in the cold, whereas the nauplii were ground in cold $HClO_4$ by the use of a glass tissue grinder. The homogenate was transferred to a Waring Blendor and extracted with gentle stirring for another 20 min in the cold, after addition of 50 ml of cold 1 N $HClO_4$. After centrifugation at 23,000 $\times$ *g* for 20 min, the supernatant fraction was poured through a charcoal column (1 $\times$ 3 cm). The column was washed with 200 ml of H_2O, and the nucleotides were eluted with approximately 300 ml of a mixture of absolute ethanol, concentrated NH_4OH, and H_2O (2:1:2, v/v/v). The nucleotide fraction was concentrated in a rotary evaporator under reduced pressure to remove ethanol and NH_4OH. The sample was made 0.002 M with respect to NH_4HCO_3, pH 8.6, and an aliquot was removed for optical density measurements at 10-mμ increments from 220 mμ to 290 mμ in a Zeiss spectrophotometer. The remainder of the sample was applied to a DEAE-cellulose column as described below.

Separation of Acid-soluble Nucleotides by DEAE-cellulose Column Chromatography—DEAE-cellulose (Whatman DE II) was prepared according to Peterson and Sober (4) and was converted to the HCO_3^- form by washing with 2 M NH_4HCO_3, pH 8.6, and was finally equilibrated with 0.002 M NH_4HCO_3, pH 8.6. The acid-soluble nucleotide fraction was percolated through a DEAE-cellulose column (1 $\times$ 20 cm), in the HCO_3^- form, and washed with 250 ml of 0.002 M NH_4HCO_3, pH 8.6. The nucleotides were eluted by use of a linear salt gradient. The mixing chamber contained 0.002 M NH_4HCO_3, pH 8.6 (1.5 liters), and the reservoir 0.25 M NH_4HCO_3, pH 8.6 (1.5 liters) (5). Elution of the nucleotides was followed spectrophotometrically at 260 and 280 mμ, and the concentrations of the individual nucleotides were calculated with the use of the appropriate extinction coefficients (6).

Isolation and Separation of Purines—The acid-soluble nucleotide fraction was applied to a charcoal column (1 $\times$ 3 cm) which was washed and eluted as described above. The nucleotide fraction was heated to boiling to remove ethanol and ammonia and to concentrate the sample, adjusted to 2 N with respect to HCl, and heated for 1 hour at 100°. After cooling, the entire fraction, which contained free purines and pyrimidine nucleotides, was applied to a Dowex 50-H^+ column (1 $\times$ 10 cm); the separation of guanine and adenine from each other and from the pyrimidine nucleotides was achieved by elution with 2 N HCl (7).

To achieve isolation of nucleic acid purines, the acid-insoluble precipitate remaining after extraction of the acid-soluble fraction was neutralized with 1 M ammonium acetate, and lipids were removed by treatment with ethanol-ether (3:1) at 45°. The nucleic acids were extracted from the lipid-free precipitate by use of a hot NaCl procedure (8) and were recovered by ethanol precipitation. The free purines and pyrimidine nucleotides were obtained by acid hydrolysis and separated by column chromatography as described above.

Extraction and Estimation of Protein—The residue remaining after removal of the acid-soluble nucleotides and nucleic acids was extracted according to the procedure of McLean *et al.* (9), and protein was separated from insoluble chitin by centrifugation. Protein concentrations were determined by a modified biuret procedure (10).

Incorporation Studies—Nauplii obtained under sterile conditions were placed in small covered dishes which contained 25 ml of sterile artificial seawater, and either $NaH^{14}CO_3$ (specific activity, 28 mC per mmole), sodium formate-^{14}C (specific activity, 44.5 mC per mmole), or glycine-2-^{14}C (specific activity, 17.1 mC per mmole) was added to a final concentration of 4 μC per ml of incubation medium. When all three purine precursors had been used in combination, each was added to a final concentration of 4 μC per ml of incubation medium. After incubation at 25° for 4 hours, the nauplii were collected and washed, and the nucleotide, nucleic acid, and protein fractions were prepared as described above. All radioactivity determinations were made with the use of a Packard liquid scintillation counter.

RESULTS

Concentration of Acid-soluble Guanine Compounds at Various Stages of Development—Table I shows the concentrations of individual guanine compounds during the development of *Artemia salina*. No significant changes occur in the amounts of guanine compounds from immersion of the cysts in artificial seawater through hatching of the nauplii. However, as the free-swimming nauplii mature a dramatic alteration takes place. Compared to the encysted embryos and newly hatched nauplii, the diguanosine tetraphosphate in the 3-day nauplii shows a 4-fold decrease. In addition, GDP decreases to a barely detectable level, whereas GMP decreases 5-fold, GTP 2-fold, diguanosine triphosphate 10-fold, and guanosine about 2-fold. During this time, the acid-soluble guanine changes from about 55 μmoles of guanine per g of protein to about 14 μmoles of guanine per g of protein, principally because of the decline in diguanosine tetraphosphate.

Because there is no increase in GMP, GTP, or any other guanine-containing compound in the acid-soluble fraction, the decrease in diguanosine tetraphosphate cannot be accounted for by a simple hydrolysis to GMP and GTP through the action of its asymmetrical pyrophosphohydrolase. Unless the products of diguanosine tetraphosphate hydrolysis are channeled directly into nucleic acid synthesis, it is difficult to understand at this point why the decrease in diguanosine tetraphosphate is not accompanied by an increase in other compounds in the acid-soluble guanine pool. This point is paramount in evaluating the function of diguanosine tetraphosphate and is considered in detail below.

Excretion of Guanine Compounds—Because of the remarkable decrease in acid-soluble guanine, we investigated the possibility of the excretion of guanine by the nauplii into the medium. Our studies indicate that very little, if any, guanine is present in the medium during the time when the acid-soluble guanine pool is decreasingly drastically. It is possible, however, that some guanine is degraded to smaller excretory products that could be overlooked but, as shown below, if this occurs at all it does so only to a minor extent.

Changes in Diguanosine Tetraphosphate and Nucleic Acid

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Guanine—Table II shows the actual decrease in concentration of diguanosine tetraphosphate, the theoretical yield of guanine resulting therefrom, and the actual increase in nucleic acid guanine occurring in the nauplii. About twice as much guanine is lost from diguanosine tetraphosphate as is found in nucleic acid guanine. If we assume that GTP produced by hydrolysis of diguanosine tetraphosphate is utilized exclusively for nucleic acid synthesis, we still cannot account for the other guanine residue. Our inability to account quantitatively for the decrease in diguanosine tetraphosphate by an increase in other guanine derivatives in either the acid-soluble pool or the nucleic acids and our inability to account for the remarkable over-all loss of guanine compounds from the acid-soluble pool force us to consider the possibility that guanine compounds are being converted by nauplii to non-guanine-containing compounds, and perhaps to other purines.

TABLE I

Concentration of acid-soluble guanine compounds at various stages of development

Acid-soluble fractions were prepared from developing cysts or from nauplii at the times indicated. After charcoal adsorption, elution, and subsequent concentration, samples were applied to and eluted from DEAE-cellulose columns (1 × 20 cm) as explained in Fig. 4 and under "Experimental Procedure." The concentrations of individual guanine compounds were calculated from absorption at 260 mμ with the use of appropriate extinction coefficients (6) including 22,300 for diguanosine tetraphosphate and diguanosine triphosphate (Finamore and Warner, unpublished data).

Developmental stage	Concentration					
	Guanosine	GMP	GDP	GTP	Diguanosine triphosphate[a]	Diguanosine tetraphosphate[a]
	μmoles/g protein					
Encysted embryos						
4 hrs	9.4	3.5		4.2	1.5	17.3
8 hrs	9.2	2.3	1.7	3.5	2.1	16.2
18 hrs	9.0	2.4	1.8	4.1	2.1	17.2
Nauplii						
Newly hatched	8.5	2.6	1.4	4.1	2.2	17.4
1 day old	5.5	0.9	0.6	2.7	0.8	8.6
3 days old	3.7	0.5	0.008	2.2	0.2	3.7

[a] Each mole of diguanosine tetraphosphate and diguanosine triphosphate contains 2 moles of guanine.

TABLE II

Changes in diguanosine tetraphosphate and nucleic acid guanine in nauplii

Data obtained from DEAE-cellulose column chromatography of acid-soluble fraction as described in Table I compared with data obtained from Dowex 50-H^+ column chromatography of nucleic acid hydrolysates as described under Fig. 1.

Time after hatching	Diguanosine tetraphosphate	Theoretical increment of nucleic acid guanine[a]	Actual increment of nucleic acid guanine
days	*μmoles/g protein*	*μmoles/g protein*	*μmoles/g protein*
0	17.4		
1	8.6	17.6	8.9
3	3.7	9.8	4.6

[a] For each mole of diguanosine tetraphosphate utilized, we assumed that 2 moles of guanine are liberated.

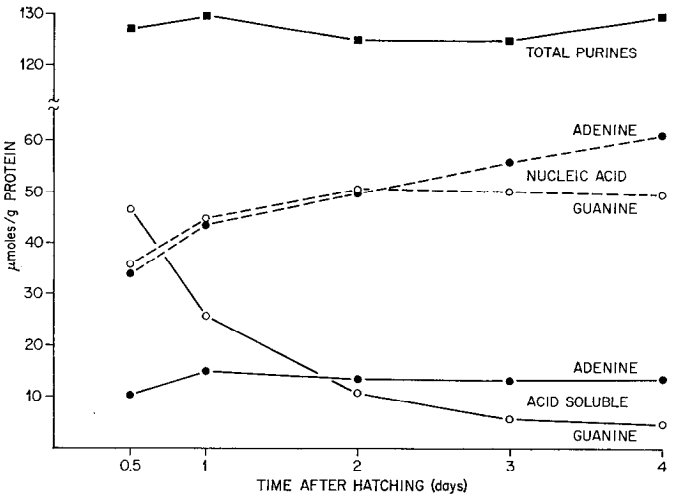

FIG. 1. Changes in total purines, acid-soluble adenine and guanine, and nucleic acid adenine and guanine in nauplii. Nauplii were collected at the times indicated after hatching, and the acid-soluble and nucleic acid fraction was extracted as described in text. After hydrolysis in 2 N HCl at 100° for 60 min, the preparations were applied to Dowex 50-H^+ columns (1 × 10 cm) and eluted with 2 N HCl to separate the adenine and guanine from each other and from the pyrimidine nucleotides (7). The concentrations of adenine and guanine were calculated with the use of the appropriate extinction coefficients (6).

Changes in Adenine and Guanine in Nauplii—Fig. 1 shows the quantitative changes occurring in the nauplii in total purines, acid-soluble adenine and guanine, and nucleic acid adenine and guanine. After hatching, the total purine concentration in the nauplii is *constant*, but significant shifts occur in the concentrations of adenine and guanine. In the acid-soluble pool, there is a slight increase in the concentration of adenine between 0.5 and 1 day after hatching, but then it remains relatively constant through 4 days. Acid-soluble guanine, as noted before, shows a marked decrease through this entire period. In addition, both nucleic acid adenine and guanine show an increase up to 2 days after hatching, after which nucleic acid guanine apparently remains constant, and nucleic acid adenine continues to increase through 4 days after hatching.

Fig. 2 shows the quantitative changes occurring in total adenine and total guanine at a time when the over-all purine level is relatively constant. It is obvious from this figure that an inverse relationship exists between total guanine and total adenine concentrations during the entire posthatch period. Table III shows the quantitative relationship between the increase in total adenine and the decrease in total guanine. These data strongly suggest that a direct conversion of guanine to adenine occurs in the nauplii of *Artemia salina*.

Synthesis de Novo of Purines by Nauplii—Since the above data indicate that guanine is used by the nauplii as a primary source of adenine, we attempted to evaluate the contribution of synthesis *de novo* to the increase in total adenine. Table IV shows, both separately and in combination, the incorporation of ^{14}C-labeled sodium bicarbonate, sodium formate, and glycine into the pyrimidines, purines, and proteins of the nauplii. Both acid-soluble and nucleic acid pyrimidines become labeled with all three precursors, and the protein fraction is heavily labeled.

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indicating adequate penetration of these precursors into the nauplii. Of primary importance, however, is the fact that virtually no radioactivity is found in either the acid-soluble purines or the nucleic acid purines, in spite of the very high levels of radioactive precursor present. That the few counts recorded in the purine fractions are most probably the result of contamination is shown in Fig. 3. This figure shows a typical elution profile of an acid-soluble nucleotide hydrolysate and a nucleic acid hydrolysate after exposure of nauplii to all three purine precursors for 4 hours. In both fractions, radioactivity is associated only with the pyrimidine nucleotides and not with the purines. Similar results have been obtained with nauplii incubation for as long as 7 hours in the presence of these purine precursors.

In view of these results, we conclude that nauplii of *Artemia salina* are unable to synthesize purines *de novo* and that they convert guanine to adenine as it is required by the organism.

Diguanosine 5'-Tetraphosphate and ATP—In our early investigations of the acid-soluble nucleotides in nauplii of *Artemia salina*, it became evident that among the acid-soluble nucleotides only ATP shows an increase concomitant with the decrease in diguanosine tetraphosphate. The increase in ATP is variable, depending on the culture conditions, but is always present. An inverse relationship is shown in Fig. 4. As diguanosine tetraphosphate decreases in the nauplii, there is a concomitant increase in ATP so that after 2 days the amount of ATP in the nauplii surpasses the quantity of diguanosine tetraphosphate.

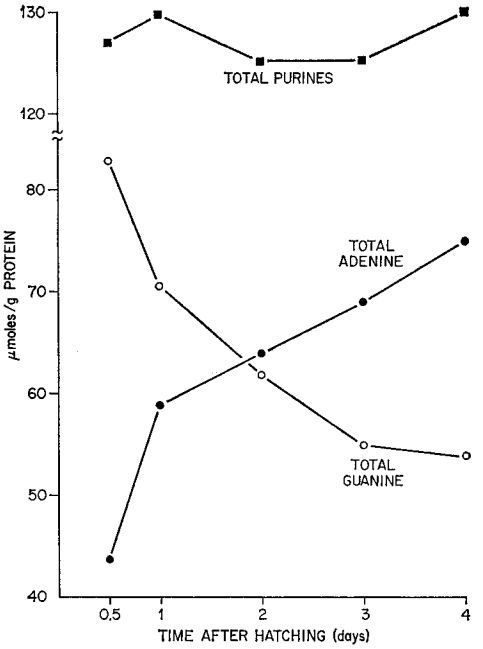

FIG. 2. Changes in total purines, total adenine, and total guanine in nauplii. Concentrations of total adenine and total guanine are obtained by combining acid-soluble adenine with nucleic acid adenine and acid-soluble guanine with nucleic acid guanine. Individual purine concentrations were determined as in Fig. 1.

TABLE III

Purine concentrations in nauplii

Concentrations of acid-soluble adenine and guanine, and nucleic acid adenine and guanine were determined from absorption at 260 mμ with the use of appropriate extinction coefficients (6) after Dowex 50-H^+ column chromatography of hydrolysates (7).

Time after hatching	Adenine				Guanine				Ratio of change in adenine to change in guanine
	Acid soluble	Nucleic acid	Total	Cumulative change in adenine	Acid soluble	Nucleic acid	Total	Cumulative change in guanine	
days	*μmoles/g protein*				*μmoles/g protein*				
0.5	10.2	34.1	44.3		46.8	35.9	82.7		
2	13.6	50.1	63.7	+18.4	10.8	50.8	61.6	−21.1	0.87
4	13.6	61.8	75.4	+31.1	4.8	49.6	54.4	−28.3	1.10

TABLE IV

Incorporation of ^{14}C-labeled bicarbonate, formate, and glycine by nauplii

Nauplii were incubated with either ^{14}C-labeled sodium bicarbonate (specific activity, 28 mC per mmole), ^{14}C-labeled sodium formate (specific activity, 44.5 mC per mmole), or glycine-2-^{14}C (specific activity, 17.1 mC per mmole) in a final concentration of 4 μC per ml of incubation medium. When all three precursors were present, each was at a final concentration of 4 μC per ml. After incubation at 25° for 4 hours, acid hydrolysates of acid-soluble and nucleic acid fractions were subjected to Dowex 50-H^+ column chromatography (7). Pyrimidine nucleotide fractions, adenine, and guanine were pooled separately, concentrated to a convenient volume, and counted. The protein fraction was extracted from the residue after removal of acid-soluble and nucleic acid fractions (9) and neutralization. All radioactivity measurements were made a in Packard liquid scintillation counter.

Isotope	Total activity						
	Pyrimidine fraction		Adenine fraction		Guanine fraction		Protein fraction
	Acid soluble	Nucleic acid	Acid soluble	Nucleic acid	Acid soluble	Nucleic acid	
	cpm/mg protein		*cpm/mg protein*		*cpm/mg protein*		*cpm/mg protein*
$NaH^{14}CO_3$	124	65	0	<1	<1	<1	436
$NaH^{14}CO_2$	204	283	<1	3	3	5	3092
Glycine-2-^{14}C	157	29	3	<1	5	6	1642
$NaH^{14}CO_3$ + $NaH^{14}CO_2$ + glycine-2-^{14}C	573	354	4	6	13	15	5415

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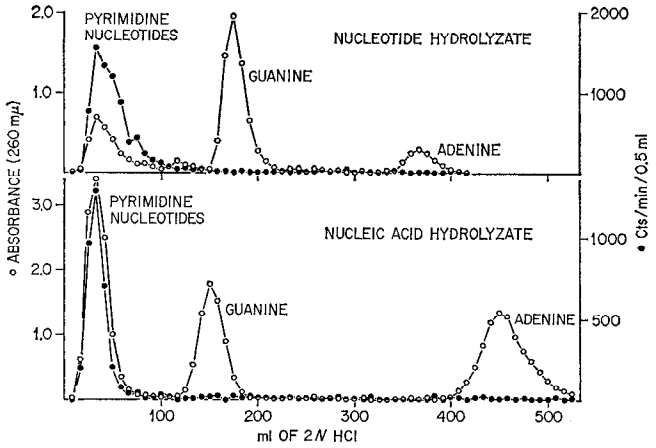

FIG. 3. Column chromatography of acid-soluble nucleotide hydrolysate and nucleic acid hydrolysate on Dowex 50-H^+. Acid-soluble fractions and nucleic acid fractions were hydrolyzed in 2 N HCl at 100° for 60 min, applied directly to Dowex 50-H^+ columns (1 × 10 cm), and eluted with 2 N HCl (7).

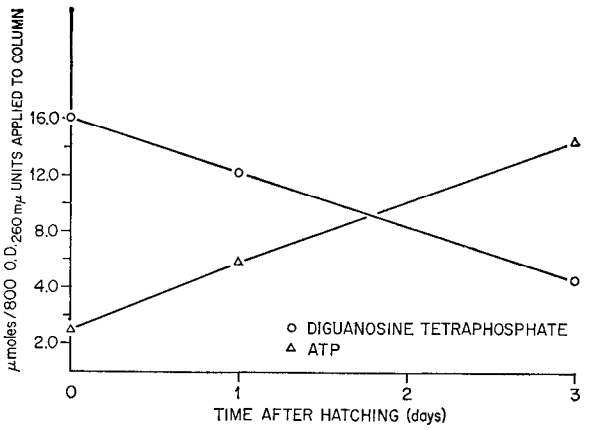

FIG. 4. Changes in P^1,P^4-diguanosine 5′-tetraphosphate and ATP in nauplii. Acid-soluble fractions, after charcoal adsorption, elution, and subsequent concentration, were applied to DEAE-cellulose columns (1 × 20 cm) in the bicarbonate form and eluted with a linear gradient from 0.002 M NH_4HCO_3, pH 8.6, to 0.25 M NH_4HCO_3, pH 8.6 (5). Concentrations of ATP and P^1,P^4-diguanosine 5′-tetraphosphate were estimated from absorbance at 260 mμ with the use of extinction coefficients of 14,200 and 22,300, respectively (Finamore and Warner, unpublished data).

This observation and the evidence that nauplii of *Artemia* convert guanine to adenine specifically suggest that diguanosine tetraphosphate is converted directly to ATP. Because ATP can be utilized for a variety of energy-requiring reactions as well as for nucleic acid synthesis, we attempted to equate the decrease in diguanosine tetraphosphate with the increase occurring in the total adenine of the nauplii. Such a calculation is shown in Table V. If we assume that for each mole of diguanosine tetraphosphate utilized 2 moles of purine are made available, then the theoretical increment in total adenine obtained from diguanosine tetraphosphate agrees with the actual increment of total adenine in the nauplii.

These results suggest that, of the acid-soluble guanine compounds present in nauplii of *Artemia salina*, diguanosine tetraphosphate may be the principal compound converted to adenine derivatives.

If this is true, how does one account for the decrease in the other acid-soluble guanine compounds? Table VI shows the decrease in acid-soluble guanine compounds (omitting diguanosine tetraphosphate) and the increase in nucleic acid guanine during the same time. The decrease in acid-soluble guanine agrees with the increase in nucleic acid guanine. These results are consistent with the postulates that diguanosine tetraphosphate is the main source of both acid-soluble and nucleic acid adenine and that other acid-soluble guanine compounds are the main source of nucleic acid guanine.

TABLE V

Changes in diguanosine tetraphosphate and total adenine in nauplii

Results of DEAE-cellulose column chromatography of the acid-soluble fraction compared with the results of Dowex 50-H^+ column chromatography of acid-soluble and nucleic acid hydrolysates.

Time after hatching	Diguanosine tetraphosphate	Theoretical increment of total adenine[a]	Actual increment of total adenine
days	*μmoles/g protein*	*μmoles/g protein*	*μmoles/g protein*
0	17.4		
1	8.6	17.6	14.9
3	3.7	9.8	10.5

[a] For each mole of diguanosine tetraphosphate utilized, we assumed that 2 moles of adenine derivatives are produced.

TABLE VI

Changes in other acid-soluble guanine compounds and in nucleic acid guanine

Results of DEAE-cellulose column chromatography of the acid-soluble fraction compared with the results of Dowex 50-H^+ column chromatography of nucleic acid hydrolysates.

Time after hatching	Total acid-soluble guanine[a]	Decrement of acid-soluble guanine	Increment of nucleic acid guanine
days	*μmoles/g protein*	*μmoles/g protein*	*μmoles/g protein*
0	21.0		
1	11.3	9.7	8.9
3	7.6	3.7	4.6

[a] Diguanosine tetraphosphate was omitted from calculations.

DISCUSSION

Conversion of Guanine to Adenine—Organisms differ in their ability to interconvert adenine and guanine. It appears that mammals can convert adenine to guanine but do not readily utilize guanine as a source of adenine (11). The protozoan *Tetrahymena gelii* requires guanine or guanine derivatives for growth but cannot use adenine as a sole source of purine (12). Also, a mutant strain of *Aerobacter aerogenes* cannot convert adenine to guanine but can use exogenous guanine to make adenine (13).

The argument for the direct conversion of guanine to adenine by the nauplii of *Artemia salina* rests on the following observations: (*a*) the total purine concentration remains constant in the nauplii even though (*b*) there is no synthesis *de novo* of purines and even though (*c*) the total adenine concentration increases; (*d*) there is a quantitative, inverse relationship between the decrease in total guanine and the increase in total adenine in the nauplii.

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Our ability to balance the changes in concentration of guanine with concomitant changes in adenine eliminates the possibility that guanine or its degradation products are being excreted by the nauplii to any major extent.

Furthermore, our results suggest that in the conversion of guanine to adenine the pyrophosphate anhydride, P^1,P^4-diguanosine 5′-tetraphosphate, is a potential as well as a real source of ATP in the nauplii. Of principal interest, however, is that in this conversion diguanosine tetraphosphate is not solely a "phosphagen," as is arginine phosphate or creatine phosphate, but that it is also a source of *adenine*.

It is obvious that in the proposed conversion of diguanosine tetraphosphate to ATP only two-thirds of the phosphate of the latter molecule is accounted for. At present we do not know the fate of the entire diguanosine tetraphosphate molecule, but we suggest that both guanine residues are converted to adenine because of the balance between the decrease in guanine from diguanosine tetraphosphate and the increase in total adenine. We are currently engaged in a search for the enzyme or enzymes responsible for this conversion.

The inability of nauplii to synthesize purines *de novo* and the utilization of diguanosine tetraphosphate as a source of ATP have compelled us to investigate the following points:

1. Whether the adults of *Artemia salina* are capable of synthesizing purines *de novo* or whether they depend entirely on an exogenous source of purines. It is of interest that this organism, when cultured under axenic conditions, requires the addition of a nucleic acid hydrolysate to the medium (14).

2. The advantage, if any, of using diguanosine tetraphosphate as a source of ATP instead of diadenosine tetraphosphate (15).

3. The mechanics of the conversion of diguanosine tetraphosphate to ATP and the determination of all of the products formed during the reaction.

4. The relationship between nucleic acid synthesis and the acid-soluble nucleotides, particularly diguanosine tetraphosphate.

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